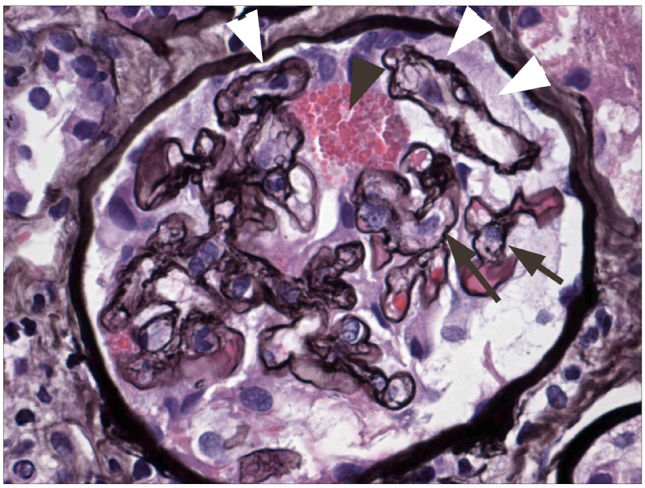
Supplementary Appendix

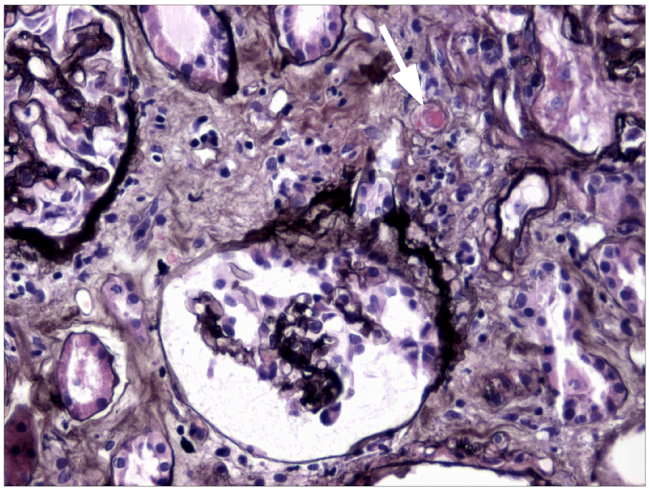
This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Eremina V, Jefferson JA, Kowalewska J, et al. VEGF inhibition and renal thrombotic microangiopathy. N Engl J Med 2008;358:1129-36.

Supplementary Figure 1



Silver stain shows focal endotheliosis with splitting of the glomerular basement membrane (white arrowheads), focal mesangiolysis (arrows) and protein droplets (black arrowhead).



Silver stain shows ischemic collapse of a glomerulus due to a thrombus (white arrow) in the afferent arteriole.

Biopsy from Patient 5 showed classic features of TMA (left hand side). Ischemic collapse of some glomeruli with thrombi in the afferent arterioles was seen. Podocyte hypertrophy is evident in the glomerulus on the left.

Supplemental Data 1

METHODS

Generation of Transgenic Mice

Efficiency of this system was tested by breeding the tetO-CMV-Cre/podocin-rtTA mice to the Z/EG reporter strain as reported elsewhere[1] and shown in Supplemental Figure 3. The degree of excision of VEGF was determined by in-situ expression analysis. Doxycycline was administered for a total period of 2 weeks.

Phenotypic analysis. Daily urinalysis was performed prior to, during and after induction with doxycycline as described.[2] Two μl of urine was run on an SDS PAGE gel on weekly urine samples as described[2]. Urinary creatinine concentration was measured using a picric acid assay according to manufacturer's instructions (The Creatinine Companion, Exocell, Inc., Philadelphia, PA, USA). Urinary albumin excretion was measured using an indirect competitive ELISA according to the manufacturer's instructions (Albuwell M, Exocell, Inc., Philadelphia, PA, USA). A total of n = 62 induced mutant mice, n = 62 induced controls (Cre negative, or heterozygous for VEGF) and n = 62 uninduced mutant transgenic mice were studied. Intra-arterial pressure measurements were performed as described[3] at 3 weeks post-induction (n = 10 mutant and n = 5 control mice) and at 5 weeks post-induction (n= 15 mutant and n = 18 control mice).

Histologic, In situ and Immunohistochemical Analysis.

Kidney tissue preparations for histologic analysis, in-situ hybridization for WT-1 and VEGF, IHC and EM were described previously[2]. Immunohistochemical staining with a rabbit polyclonal antibody directed against fibrinogen (1:3000 dilution, Dako, Carpenteria, CA, USA) was performed on dewaxed paraffin tissue sections that had been pretreated with protease I, for 16 minutes and blocked for endogenous peroxidase and biotin. Streptavidin-biotin complex immunodetection was performed using the Ventana iView Open secondary DAB (3-3'-diaminobenzidine) Detection System. The secondary antibody was biotinylated goat anti-rabbit IgG (1/100 dilution; Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin.

Statistical analysis

A non-parametric Wilcoxon rank sum test was used. Results are expressed as the median +/- standard deviation (SD) for blood pressures or mean +/- SD for albumin:creatinine ratios.

Contributions:

Patient clinical histories (1-4) were provided by Dr. Jefferson, Dr. Kowalewska, Dr. Richardson and Dr. C. Alpers. Dr. C. Alpers interpreted pathology of kidney biopsies. Case history of patient 5 was provided by Dr. Hochster and Dr. Weisstuch and pathology interpreted by Dr. L. Barisoni. Dr. Haas provided the clinical history and pathology of patient 6. The first draft of the manuscript was written by Dr. Quaggin and edited by all of the co-authors. Mouse genetic studies were designed and interpreted by Dr. Quaggin and Dr. Eremina. Dr. Alpers interpreted mouse renal pathology. Blood pressure measurements were performed by Dr. Kabir and Dr. Eremina and data interpreted by Dr. Eremina, Kabir, Backx and Quaggin.

References:

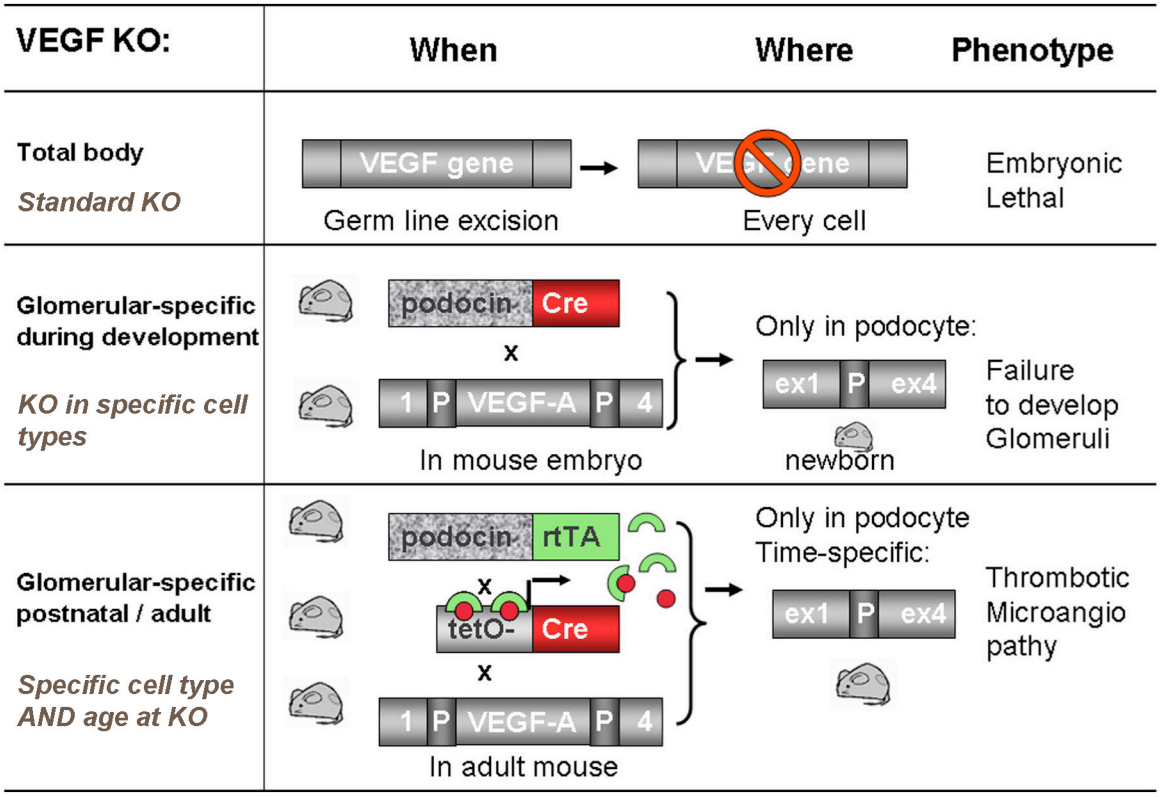
1. Belteki G, Haigh J, Kabacs N, et al. Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. Nucleic Acids Res 2005;33(5):e51.

2. Eremina V, Sood M, Haigh J, et al. Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. J Clin Invest 2003;111(5):707-16.

3. Zvaritch E, Backx PH, Jirik F, et al. The transgenic expression of highly inhibitory monomeric forms of phospholamban in mouse heart impairs cardiac contractility. J Biol Chem 2000;275(20):14985-91.

Supplementary Figure 2

Evolution of the Knockout Mouse



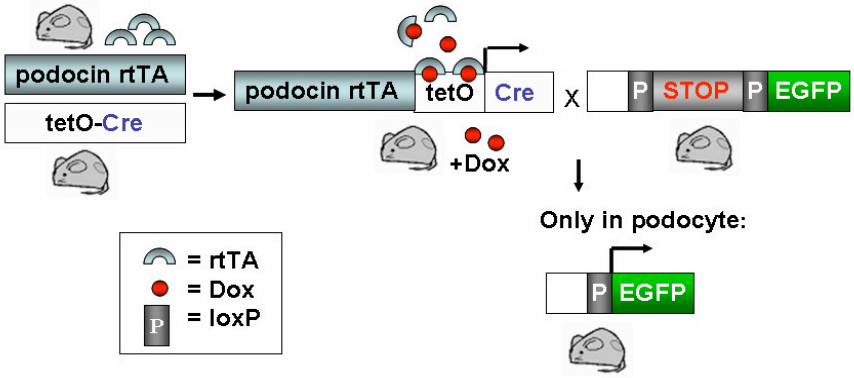
Standard knockout results in germline deletion of the VEGF gene (KO in all cell types from initial stage of conception). Homozygotes and heterozygotes die at embryonic day 9.5 and 11.5, respectively, due to failure of vascular growth.

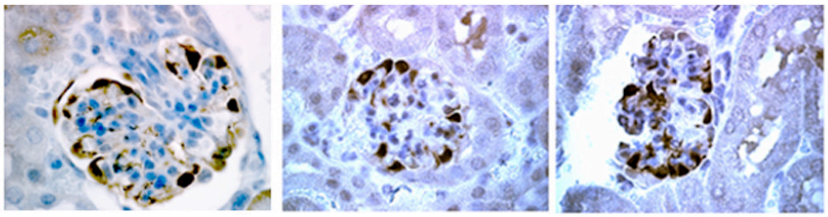
Cell-specific knockouts are accomplished using the Cre-loxP gene targeting system. A Cre – deletor strain of mouse expresses the Cre-recombinase enzyme in a cell type of interest (e.g. the podocyte, under regulation of a podocyte-specific promoter from the podocin gene). A critical exon of the VEGF gene is 'floxed'; loxP sites that are 34-bp repeats are inserted in non-functional parts of the VEGF gene. Upon breeding with the Cre-deletor strain, Cre-recombinase causes site specific recombination between loxP sites and 'loops out' or 'knocks out' the VEGF gene ONLY in podocytes.

To accomplish both time and cell-specific knockouts, a third component must be introduced to the system. Here, the reverse tetracycline transactivator protein (rtTA) is expressed under

control of a podocyte-specific promoter. This mouse constitutively expresses rtTA in its podocytes, but has no phenotype. This mouse is bred to a mouse that contains Cre-recombinase under control of a 'tetO' promoter from CMV (cytomegalovirus). In the absence of tetracycline or a tetracycline analog (e.g. doxycycline), NOTHING happens. When doxycycline is added to the drinking water or food of the mouse, the doxycycline combines with the rtTA protein and acts as a 'genetic switch', binding to the tetO-promoter and turning on expression of Cre recombinase. When these 2 transgenes are carried in the same genome as a mouse carrying 2 floxed VEGF alleles, the VEGF gene is knocked out. This permits the researcher to control the knockout in TIME and SPACE.

Supplementary Figure 3



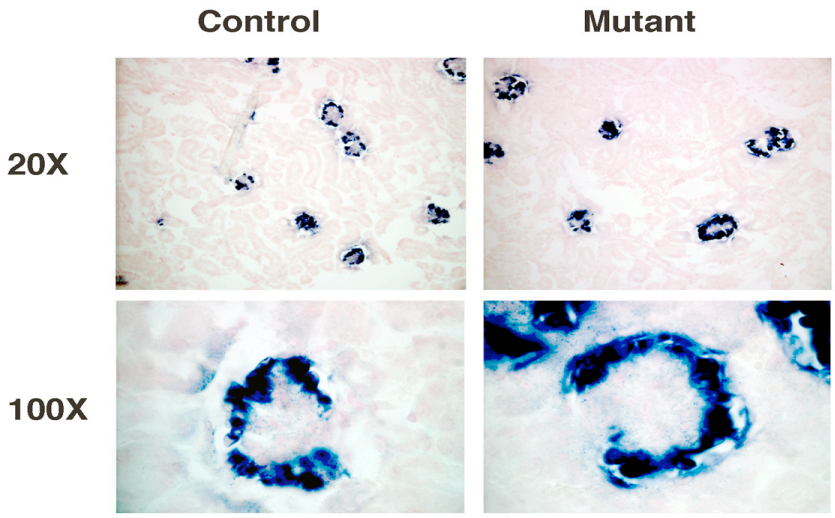


EGFP experssion is only observed in podocytes

Validation of the Cell and Time Specificity of the Tet-ON System in Podocytes

To determine the degree and specificity of Cre-mediated excision using the podocyte-specific TetON system, a mouse carrying the podocin-rtTA transgene and a tetO-Cre transgene was bred to a mouse carrying the Z/EG reporter transgene. Triply transgenic mice were examined with and without doxycycline. In the absence of doxycycline, no excision occurs, and there is no expression of enhanced green fluorescent protein (EGFP) in any cell type. When doxycycline is added to the drinking water or food of the triply transgenic mouse, doxycycline and the reverse tetracycline transactivator (rtTA) protein (that is only expressed in podocytes) forms a complex that binds to the tetO promoter regulating expression of Cre recombinase. This acts as a 'genetic switch' to turn on Cre, leading to excision of the floxed STOP codon (excision of the STOP codon flanked by loxP sites) and expression of the EGFP protein. 80 to 90 % of podocytes express the EGFP protein. No other cells in any other organ turn green, demonstrating that this system is time and podocyte-specific.

Supplementary Figure 4



Nephrin expression

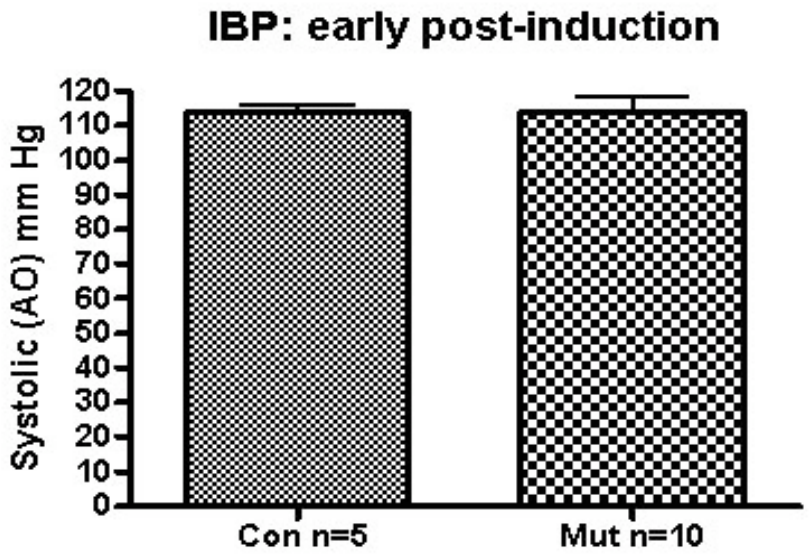
A

Definition	Gene Symbol	Fold Change Mut vs Con
podocin	Nphs2	+1.3
nephrin	Nphs1	+1.3
WT1	WT1	+1.1
nestin	Nes	+1.1
actinin alpha 4	Actn4	+1.2

B

A. In situ analysis for Nephrin RNA expression was performed on control and VEGF mutant kidneys. At the onset of proteinuria, no differences were observed. **B.** Gene expression profiling was also performed; probes were generated from RNA isolated from sieved glomeruli (mutant vs. control) and hybridized to the Illumina Sentrix Mouse-8 Bead Chip microarray chips (n = 4 mutants and n = 4 controls, 8 experiments). These studies confirmed that podocyte-expressed genes such as nephrin (NPHS1), podocin (NPHS2), Wilms tumour suppressor 1 (WT-1), Nestin (Nes) and alpha actinin 4 were unchanged in glomeruli of mutant mice at the onset of proteinuria.

Supplementary Figure 5



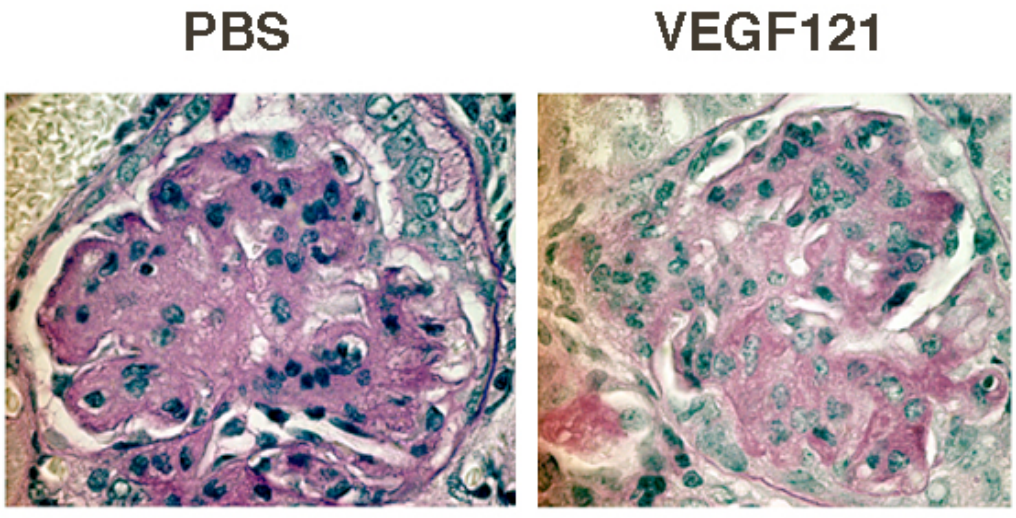
Blood Pressure Measurements at Onset of Proteinuria Were Not Different Between Groups

The means of intraarterial systolic blood pressure measurements are shown. Mutant mice were induced at 3 weeks of age with doxycycline while control mice carried the same genotype but received no doxycycline. Measurements were performed 3 - 4 weeks post-induction. At this time, mutant mice exhibited 1+ or 2+ proteinuria and early glomerular changes on light microscopy. Mean blood pressures +/- standard deviation measured: 113.7 +/- 15.0 in mutants and 113.7 +/- 5 mm Hg in controls ($p = 0.85$, Wilcoxon rank sum test).

Supplementary Data 2

Systemic Administration of VEGF-121 fails to rescue the phenotype

Human VEGF121 (kind gift of A. Karumanchi, Harvard University) or PBS (placebo/carrier) was administered at a dose of 50 μg/kg by subcutaneous injection twice daily as previously described [1]. This dose and isoform were chosen as it had previously been reported to improve renal outcome in rats with TMA[1]. Mice were observed daily and urinalysis performed weekly. A total of 80 mice were studied (n = 20 in each group: dox + PBS, no dox + PBS, dox + VEGF121, no dox +VEGF121). Mice were sacrificed 4 weeks following start of treatment and kidneys processed for histology. Samples were scored histologically; the pathologist was blinded to the genotype and treatment of the mice. Histologic scoring of the glomerular lesion (mesangial matrix expansion, glomerulosclerosis, crescent formation) was performed as described.[2] No improvement in histologic score was observed in mice treated with 100 ug/kg/day VEGF121.



Representative glomeruli from mutant mice induced with doxycycline (VEGF podocyte-selective knockout mice) and treated with placebo (PBS) or VEGF121. No difference was observed by histologic scoring.

References:

1. Suga S, Kim YG, Joly A, et al. Vascular endothelial growth factor (VEGF121) protects rats from renal infarction in thrombotic microangiopathy. Kidney Int 2001;60(4):1297-308.
2. Raij L, Azar S, Keane W. Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. Kidney Int 1984;26(2):137-43.